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Effects of different types of oxidative stress (OS) on nuclear signal transduction were compared. Human blood lymphocytes were subjected to: a) Acute exposure to H_2O_2; b) Chronic exposure to H_2O_2; and c) Ionizing radiation, and then activated for 6 hours. DNA-binding activities of 3 transcription factors (TF): NFκB, AP-1 and NFAT, were abolished in T lymphocytes by all 3 types of OS. Two other TF, AP-3 and NRE-A, were mostly unaffected. Radiation exposure was also studied in B cells and had the same effect as on T cells. Epithelial cells transfected with NFkB- and AP-1-directed chloramphenicol acetyl transferase (CAT), expressed significantly less CAT upon IL-1 stimulation when pre-exposed to H_2O_2. Neither T nor B lymphocytes exhibited significant lipid peroxidation upon exposure to OS.

Conclusions: 1) Suppression of specific TF functions can serve as a marker of lymphocyte exposure to OS; 2) This functional measure is more sensitive to OS and longer-lived than lipid peroxidation; and 3) Based on these results, an *in vitro* screening assay to identify oxidants, and an assay to monitor human exposures to oxidants, can be developed.

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INTRODUCTION

Lymphocyte activation and signal transmission. T lymphocytes are activated following the binding of a ligand to the antigen receptor complex (CD3/Ti). One of the early manifestations of this interaction is the transcriptional activation of the IL-2 gene[1] which requires simultaneous activation of protein kinase C and elevation of intracellular Ca^{2+} concentration[2]. IL-2 is a pivotal lymphokine involved in B and T lymphocyte, as well as natural killer cell regulation[1]. An immediate consequence of T cell activation is the phosphorylation of a wide range of proteins[3,4]. The modulation of IL-2 transcription by nuclear factor(s) can serve as a general readout that would be affected by any abnormality occurring earlier in the pathway. The promoter region of the IL-2 gene, in the 5′ flanking region, controls induced T cell specific gene expression[5]. A transcriptional enhancer lies in this region and responds to signals generated after activation through the T cell antigen receptor[6].

A number of positive regulatory elements have been identified in this region, including: NFAT, AP-1, NFkB and EGR-1. The activities of proteins capable of binding to these sequences and thereby enhancing the expression of the IL-2 gene (transcription factors), appear only in activated human T cells[7-9]. Other transcription factors, expressed in human T lymphocytes, bind to the following sequences in the IL-2 promoter: AP-3, Oct-1, and Sp1. NFAT-1, AP-1 and NFkB, which are only expressed in primary human T cells following stimulation, are plausible targets for suppression of T cell activation. A zinc finger containing protein that inhibits IL-2 gene expression has also been described. A nucleotide sequence (NRE-A) that binds to this protein has been identified between -101 and -110[10].

B lymphocytes do not express the IL-2 gene but do exhibit activities of some of the transcription factors that regulate the expression of IL-2 in T cells. In B cells, these transcription factors are involved in the enhancement of the expression of other genes such as immunoglobulin genes. B cells express Oct-1, NFkB and AP-1 activities[11-13]. NFAT DNA-binding activity can also be detected in B cells[13] although it does not appear to enhance gene expression in human B cells[14].

Environmental oxidative stress. A variety of distinct biochemical changes in lymphocytes and in various other target cells are induced by oxidants, e.g., hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). These changes include alterations in enzymatic activities, lipid peroxidation and damage to DNA. H_2O_2 rapidly permeates cells and would, in most cellular environments, have a lifetime that would permit it to diffuse appreciable distances before reaction. It is therefore proposed that H_2O_2 is the dominant oxidant leading to DNA strand breaks[15]. Also, oxidizing reactive species on free-radical-damaged proteins, protein hydroperoxides that can consume glutathione, have been demonstrated. The long-lived nature of the reactive moieties indicates that they may be able to diffuse and transfer damaging reactions to distant cellular sites[16].

The principal oxidants in the lower atmosphere are ozone and two by-products of ozone photodissociation, the hydroxyl radical and hydrogen peroxide[17]. Ozone is a very toxic air pollutant affecting organic molecules via free radical- and lipid peroxide-mediated mechanisms[18]. T lymphocytes from subjects exposed to ozone *in vivo* exhibited significantly reduced mitogenic response for several weeks following the exposure[19,20]. Since ozone is a highly reactive oxidant pollutant, it is unlikely that it interacts directly with blood lymphocytes. Rather, the lymphocytotoxic effect of ozone is probably mediated by soluble products of pulmonary cells that are affected directly by the oxidant[21]. Pryor et al.[22] have demonstrated that the interaction of ozone in the presence of water with unsaturated fatty

acids, ozone's primary target in lung lining fluids, produces aldehydes and hydrogen peroxide. When bronchoalveolar lavage (BAL) was used, the yield of hydrogen peroxide production was 55%. Based upon those experiments with ozone at 2.8 ppm (nearing smog levels) the interaction with BAL would yield approximately 8.25 nmoles/ml H_2O_2 over two hours. The polyamine oxidase system we studied involves oxidative stress exerted on lymphocytes by enzymatic products, which include hydrogen peroxide and an aldehyde, at about 5 nmoles/ml H_2O_2 over forty eight hours. This exposure approximates the daily average of urban ozone profile including night time - 0.1 ppm[23]. Therefore, we propose that our system can serve as a model for environmental oxidant exposure.

Ionizing radiation can be used as a means of introducing oxygenating radicals into lymphocytes in a geometrically and temporally precise way. The absorption of radiation involves splitting H_2O molecules (the most common constituent of cells) into $OH^\bullet$ and $H^\bullet$ radicals which are initially distributed in proportion to the radiation dose distribution[24]. $OH^\bullet$ radicals generated within a cell would generally react immediately with very little diffusion into the surrounding medium. In addition irradiation of dissolved O_2 will produce the superoxide radical, $HO_2^\bullet$, also following the radiation dose distribution. The superoxide radical has intermediate reactivity between that of $OH^\bullet$ and H_2O_2 and will diffuse moderate distances before reacting. In comparison, H_2O_2 is less active and may diffuse many cell diameters before interacting with cellular macromolecules. Accelerated electrons are quite easy to control and precise levels of oxidative stress can be generated by irradiating cells and the surrounding medium. A radiation dose of 1.0 Gy will generate 2.72×10^{-8} mol/l of $OH^\bullet$, 0.68×10^{-8} mol/l of H_2O_2 and 0.008×10^{-8} mol/l of $HO_2^\bullet$ in water, and similar values are expected initially in the water component of cells growing in nutrient medium. The use of ionizing radiation to administer oxidative stress enhances the control of the concentration and timing of the exposure and provides a different type of stress (mainly mediated by $OH^\bullet$) than that produced by enzymatically-generated H_2O_2.

Polyamine oxidation and IL-2 biosynthesis. We have previously described a new mechanism of IL-2 down-regulation[25]. Endogenous H_2O_2 produced by monocytes and endogenously produced or exogenously added polyamines all provide down-regulatory signals for IL-2 production by human peripheral blood T cells. The interaction between polyamine oxidase (PAO) and the polyamine spermidine generates products (including H_2O_2) that suppress IL-2 production. PAO activity suppressed protein tyrosine phosphorylation and calcium mobilization. If indeed the effects of PAO on early signaling events cause reduced IL-2 biosynthesis then we would expect to detect those effects later in the signaling cascade, reflected in the expression of transcription factors that regulate the IL-2 gene.

Hypothesis

Oxidative stress alters the expression of transcription factors in human lymphocytes. Given the critical role these factors play in gene regulation, their abnormal regulation should lead to disturbances in gene expression and these in turn will result in cellular dysfunction. The transcription factor abnormalities may be used to develop an assay for the detection of environmental toxic oxidants.

Technical objectives

To study three models of inducing oxidative stress in lymphocytes: a) PAO activity generating extracellularly low levels of H_2O_2 for two days (mimicking exposures to environmental chemical toxicants); b) electron irradiation generating both extra- and

intracellularly mainly $OH^{\bullet}$; and c) high levels of reagent H_2O_2 generating short but acute stress. The following questions were asked during the first year of work:

Do human lymphocytes subjected to oxidative stress: a) exhibit abnormal cellular function-expression of transcription factors that regulate the interleukin 2 gene (essential for T lymphocyte function)?; and b) sustain damage as assessed by an independent and established method: measurement of lipid peroxidation?

BODY

Methods

T and B cells from the peripheral blood of healthy donors were studied and the human epithelial cell line - A549 was used for the transient transfection assays. Cells were incubated for all assays in a serum-free medium, because fetal calf serum contains PAO activity[25]. Therefore, RPMI-1640 with Nutridoma-HU supplement (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used.

Lymphocyte preparation. Heparinized peripheral blood from healthy donors was used as a source of lymphocytes. Cells were purified by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. The resultant mononuclear cell preparation was allowed to adhere to plastic dishes to remove macrophages and other adherent cells. Non-adherent mononuclear cells were then used either as a source of T or B cells.

For T cells, non-adherent mononuclear cells were mixed with a suspension of neuroaminidase-treated sheep erythrocytes and incubated at 37°C for 15 min, followed by centrifugation and further incubation at 4°C for 45 min. Thereafter, the rosetted cells were obtained by centrifugation through Ficoll-Hypaque. The erythrocytes in the cell pellet were lysed by exposure to 0.83% NH_4Cl. The rosetted cells contained more than 98% $CD3^+$ T cells, and 0.4-1% $M3^+$ monocytes as determined by flow cytometry.

For B cells, non-adherent mononuclear cells were mixed with magnetic beads carrying anti-B cell antibodies on their surface and incubated at 4°C for 15 minutes. Dynabeads M-450 with anti-CD19 antibodies (Dynal, Lake success, NY) were used at ten million beads/ml. The rosetted CD19+ B cells were isolated by magnetic force and the beads were detached using reagents and equipment from Dynal. The resultant isolated B cells were unstimulated and 99% pure, as determined by flow cytometry.

The A549 lung epithelial cell line was obtained from ATCC (Rockville, MD) and passed in F-12 K + 10% fetal calf serum at 0.5-1x10^6/ml, 37°C, 7.5% CO_2.

Oxidative stress. These modes were used:

a) A longitudinal low level extracellular stress (mimicking exposures to environmental chemical toxicants) - lymphocytes were preincubated for 2 days with a commercial preparation of PAO (Sigma) at 5x10^{-4} U/ml and spermidine at 5 μM. This exposure generates gradually 5 μM H_2O_2 over two days[25].

b) Electron radiation generating both extra- and intracellularly mainly $OH^{\bullet}$ - lymphocytes were exposed to a radiation dose of 6 Gy for 5 minutes. This dose produces nonlethal cellular responses[26] and generates oxidants per time unit at about 20 fold higher levels than mode a), but for a much shorter period of time. We used a 2.5 MeV Van de Graaff accelerator that is capable of generating electrons or protons to a maximum energy of 2.5 MeV. Doses were

continuously monitored by means of parallel plate ionization chambers coupled with a stable, vibrating reed, electrometer.

c) A short high level extracellular stress-reagent H_2O_2 was added directly at 20, 50,100 and 200 μM for 2 hours. We have found that these levels suppress IL-2 production in human blood lymphocytes by 17, 67 and 88%, respectively, without affecting cell viability[27].

The suppressive effect was studied in the preincubation mode in order to exclude any possible effects on the assay used. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Measurements of transcription factor activities. For T cell activation we used PHA (1 μg/ml) + TPA (5 ng/ml), and for B cell activation we used anti-μ antibodies (Pharmingen, San Diego, CA; pre-coated on the bottom of 24-well plates), for 6 h at 37°C, 5% CO_2, prior to collecting the cells for nuclear extraction.

DNA-binding

Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts. Cells were washed and nuclear extracts were prepared according to a modification of Schreiber et al.[28]. This method is suitable for small numbers of cells and therefore appropriate (based on our experience, Flescher et al.[29]) for studies of peripheral blood lymphocytes. Cells were washed and resuspended in Tris buffered saline, transferred to an Eppendorf tube and repelleted. The cell pellet was resuspended in a buffer containing: 10 mM Hepes, 10 mM KCl, 0.1 mM ETDA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 μg/ml Aprotinin, 5 μg/ml Antipain, 100 μM Benzamidine, 5 μg/ml Leupeptin, 5 μg/ml Pepstatin, 5 μg/ml soybean trypsin chymotrypsin inhibitor, pH 7.9. The cells were allowed to swell on ice for 15 min and NP-40 at 0.625% was added. The tube was vortexed for 10 seconds and centrifuged for 30 seconds in a microfuge. The nuclear pellet was resuspended in a buffer containing 20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and the 7 aforementioned protease inhibitors, pH 7.9. The tube was vigorously rocked on wet ice for 15 minutes on a shaking platform and the nuclear extract was centrifuged for 5 minutes. Protein concentration of the supernatant was determined (Bradford method, Bio-Rad Protein Assay Kit). Aliquots were stored at -70°C.

DNA-protein interactions. DNA probes containing the binding sites from the IL-2 promoter region[7] were purchased from Genosys (The Woodlands, TX). The probe for NFAT-1 spans between nucleotides -255 and -285:
5′-GGAGGAAAAACTGTTTCATACAGAAGGCGTT-3′
The probe for AP-1 spans between nucleotides -140 and -156:
5′-TTCAAAGAGTCATCAG-3′
The probe for NFκB spans between nucleotides -190 and -214:
5′-TAACAAACAGGGATTTCACCTACAT-3′
The probe for AP-3 spans between nucleotides -201 and -211:
5′-AAAGAGGGATT-3′
The probe for IL-2 transcription inhibitory protein (NRE-A) spans between nucleotides -99 and -112:
5′-AATTCCAGACAGGTAAAGTGTTAA-3′

The probes were labeled with ^{32}P-ATP using T4 polynucleotide kinase (Promega). We combined 10,000 cpm DNA probe (~ 0.2 ng), 2 μg poly(dI-dC) (a nonspecific competitor DNA), 3 μg BSA (a protein carrier) and 10 μg nuclear extract in a final reaction volume of 20 μl. The binding reaction mixture was incubated for 15 min in a 30°C water bath. The protein-DNA

complexes were detected on a 4% low-ionic-strength native polyacrylamide gel. The gel was dried under vacuum and autoradiographed.

Chloramphenicol Acetyl Transferase (CAT) expression in transfected cells

To test the functional relevance of EMSA we transfected cells with constructs in which the activity of CAT is directed by DNA-binding sequences. CAT activity in those cells was measured by ELISA.

Transient cellular transfections. A549 epithelial cells (grown at $5x10^5$ cells/ml) were used as hosts for the CAT constructs containing sequences from the IL-2 promoter. Two CAT reporter vectors (J16-A Δ^{56} plasmid containing 2 copies of NFκB sites and a C-Fos promoter contact; and MAP-1 pBLCAT2 plasmid containing an AP-1 site and a minimal TK promoter, generously supplied to us by Dr. M. Lenardo of the NIH) were introduced into A549 cells via calcium phosphate coprecipitation procedures using a Mammalian Transfection kit (Stratagene, La Jolla, CA). The efficiency of DNA uptake was monitored by cotransfection with the plasmid pCH-110 containing the β-galactosidase gene driven by the SV-40 promoter (Pharmacia, Piscataway, NJ). CAT and β-galactosidase activities were measured after 48 hr of culture at 37°C using ELISA kits (Boehringer Mannheim, Indianapolis, IN). Activity was calculated per protein concentration and efficiency of transfection.

Measurements of lipid peroxidation

Quantitative peroxide assay. A lipid compatible formulation of the PeroXOquant Quantitative Peroxide Assay (Pierce Chemical Co., Rockford, IL) was used. This assay is adapted to measure cellular hydroperoxides. To differentiate between hydrogen peroxide and peroxides of cellular molecules (such as lipid peroxides) we followed the recommendations of the manufacturer and regarded any catalase (7000U/ml)-inhibitable measurement as representing hydrogen peroxide. In the assay, peroxides convert Fe^{2+} to Fe^{3+} in a sulfuric acid solution. The Fe^{3+} complexes with the xylenol orange dye to yield a purple product with absorbance at 540-600 nm. The molar extinction coefficient of the xylenol orange-Fe^{3+} complex is $1.5x10^4$ $M^{-1}cm^{-1}$ in 25 mM H_2SO_4 at room temperature. Five million cells were lysed by sonication (two 10 seconds pulses with a 10 seconds interval) and incubated for 15-20 minutes at room temperature in the following working solution (10 times the volume of the sonicate): 0.25mM ammonium ferrous (II) sulfate, 25mM H_2SO_4, 4mM BHT, 125μM xylenol orange in methanol. Results were read at 595 nm in a microtiter plate reader. For calibration and validation, a series of hydrogen peroxide solutions at concentrations between 1μM to 1mM were prepared and assayed. Results were calculated per protein concentrations as determined by the Bradford method. Since the method allows to measure peroxides without lipid extraction, a blank without ammonium ferrous (II) sulfate and H_2SO_4 was used to subtract endogenous iron, and other transition metals, interferences[30].

Statistical analysis of data. Data was analyzed, where appropriate, using student's T test.

Results

Our Statement Of Work for the first year proposes to answer two questions: 1) Does oxidative stress induce abnormal cellular function-expression of transcription factors

regulating the IL-2 gene, in lymphocytes? 2) Does oxidative stress induce lipid peroxidation in these cells?

Both questions were answered in studies on human peripheral blood T and B lymphocytes. The first question was approached in two ways: **a.** By demonstrating interactions between transcription factors and the cognate DNA sequences they bind to. **b.** By measuring the activity of a reporter gene regulated by these transcription factors.

Transcription factor DNA-binding studies

Since this study aims to develop markers of oxidative stress-induced suppression of cellular function, we studied the ability of DNA sequences from the IL-2 promoter to bind to proteins present in nuclei of lymphocytes that are stimulated by mitogens and are commencing proliferation. IL-2 is central to the cellular immune response and inability to express this gene would result in cellular dysfunction of T lymphocytes. B cells were also studied, although they do not express IL-2, because these cells use the same transcription factors for the regulation of other genes. The DNA-binding assay is based on the fact that DNA bound to a protein (the transcription factor) will move slower during gel electrophoresis and appear higher (closer to the origin) on the gel. The DNA is labeled radioactively, thereby allowing the position of the DNA-protein complex to be determined on the gel. Three DNA-binding activities present in activated lymphocytes were studied: NFκB, AP-1 and NFAT. The cells were subjected to six types and levels of oxidative stress: an enzymatic activity (PAO) generating hydrogen peroxide, irradiation and four concentrations of hydrogen peroxide administered directly to the cells. Figure 1 demonstrates the effect of oxidative stress on NFκB DNA-binding in T lymphocytes. While unstimulated cells (Unst.) do not express NFkB DNA binding, stimulated cells (Stim.) do express this activity and the interaction is specific as shown by its prevention in the presence of a specific competitor (Stim.+Comp.). All the types and levels of oxidative stress we employed completely abolished the induction of NFκB DNA-binding in stimulated T lymphocytes.

As can be seen in Figures 2 and 3, protein binding activities to two other DNA sequences (AP-1 and NFAT) were only expressed in stimulated T cells and were abolished by exposing the cells to oxidative stress, similar to the results obtained with the NFκB sequence.

Since three activation-dependent DNA-binding activities (NFκB, AP-1, NFAT) were suppressed by oxidative stress, it was necessary to determine whether oxidative stress affects every DNA-binding activity in the cells in a non-specific manner. To that end, we studied two more DNA-binding activities that are expressed in human T lymphocytes: NRE-A which is expressed constitutively, and AP-3 which was expressed only upon cellular activation. As can be seen in Figure 4, only the activity of polyamine oxidase suppressed NRE-A DNA-binding while the other types of oxidative stress had no significant effect on the appearance of the NRE-A-protein complex. Only treatment with hydrogen peroxide at the highest concentration (200 μM) fully abolished both bands of the AP-3 DNA-binding activity (Figure 5).

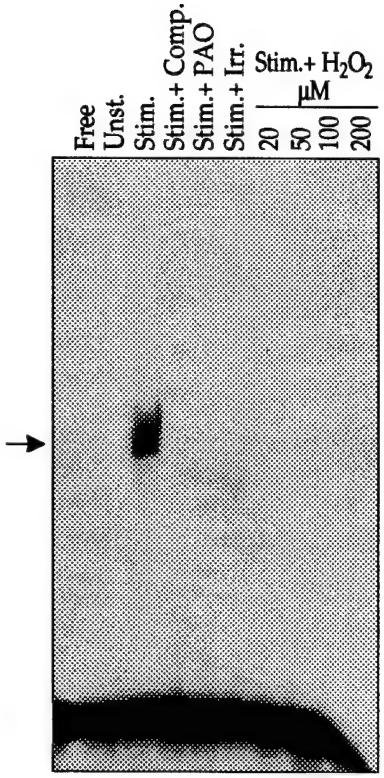

Figure 1. Suppression of NFκB DNA-binding by oxidative stress. T cells were pre-treated with polyamine oxidase (Stim.+PAO, for 2 days at $5x10^{-4}$U/ml + spermidine at 5μM and then washed and incubated for 2 hours in fresh medium), or irradiation (Stim.+Irr., for 5 minutes at 6 Gy and then incubated for 2 hours in fresh medium), or hydrogen peroxide (Stim.+H_2O_2, for 2 hours at the indicated concentration and then washed incubated for 2 hours in fresh medium). Cells were then stimulated with PHA (1μg/ml) + TPA (5ng/ml) for 6 hours. In addition, control cultures of untreated cells were either not stimulated (Unst.) or stimulated with PHA + TPA for 6 hours (Stim.). Nuclear extracts were prepared and 10 μg of protein was incubated with ^{32}P-labeled NFκB sequence and electrophoresed. The lanes were loaded with DNA without nuclear extract (Free), DNA with extract from untreated and unstimulated cells (Unst.), DNA with extract from untreated and stimulated cells (Stim.), same as Stim. + 50x excess of unlabeled probe (Stim.+Comp.), and DNA with extracts from pre-treated cells that where also stimulated (Stim.+PAO, Stim.+Irr., Stim.+H_2O_2). The gel was dried and autoradiographed. The arrow marks the specific DNA-protein complex.

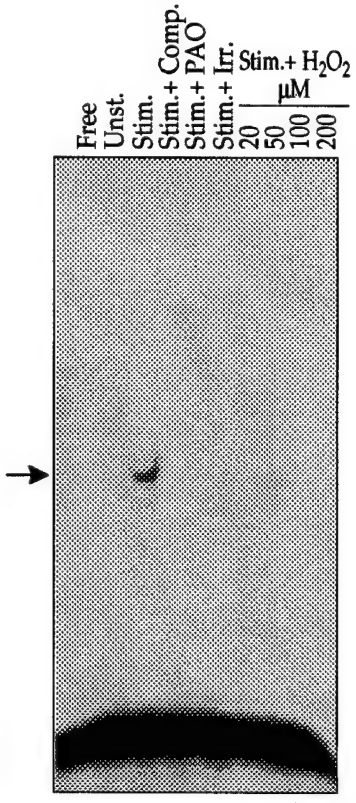

Figure 2. Suppression of AP-1 DNA-binding by oxidative stress. Same as Figure 1 except that ^{32}P-labeled AP-1 sequence was used.

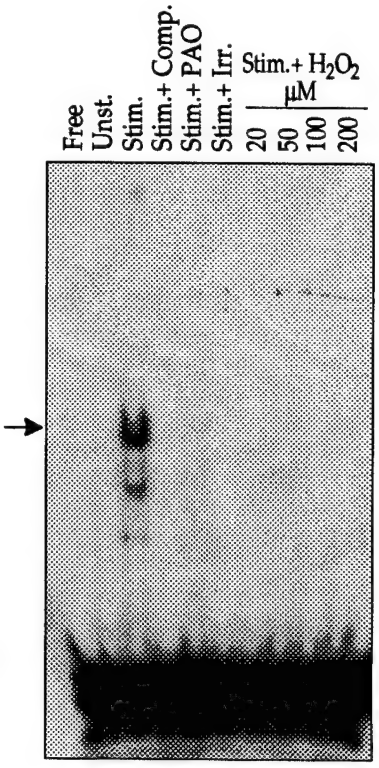

Figure 3. Suppression of NFAT DNA-binding by oxidative stress. Same as Figure 1 except that ^{32}P-labeled NFAT sequence was used.

The effect of oxidative stress on B cell DNA-binding activities was determined, using the three sequences: NFkB, AP-1 and NFAT, which did not bind cognate proteins when nuclear extracts from stressed T cells were studied. The aim was to compare the responses of B and T cells to oxidative stress. Therefore, one mode of oxidative stress (irradiation) that suppressed all three DNA-binding activities in T cell extracts, was employed in these experiments. Irradiation inhibited the expression of all three DNA-binding activities in activated B lymphocytes (Figure 6), similar to the effects of irradiation on T lymphocytes (Figures 1,2,3).

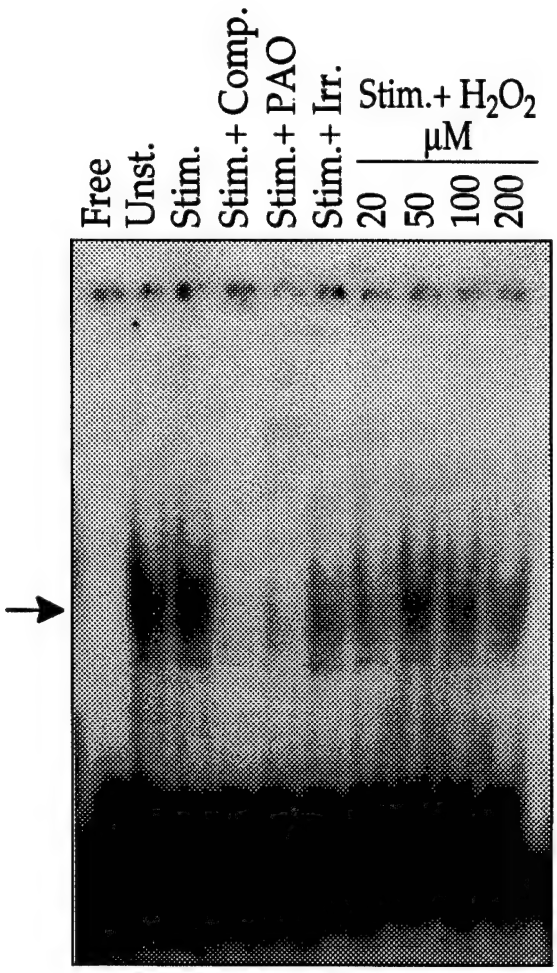

Figure 4. NRE-A DNA-binding activity in oxidatively-stressed lymphocytes. Same as Figure 1 except that ^{32}P-labeled NRE-A sequence was used.

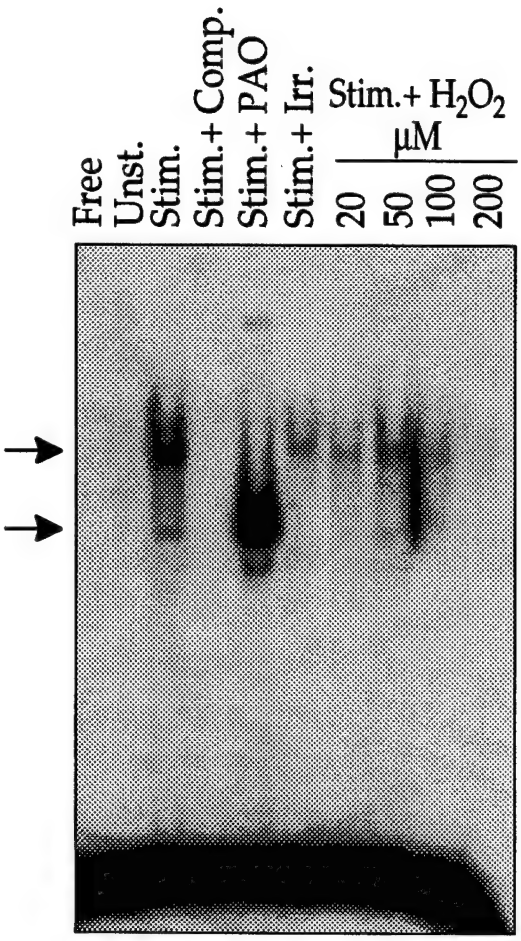

Figure 5. AP-3 DNA-binding activity in oxidatively-stressed lymphocytes. Same as Figure 1 except that ^{32}P-labeled AP-3 sequence was used.

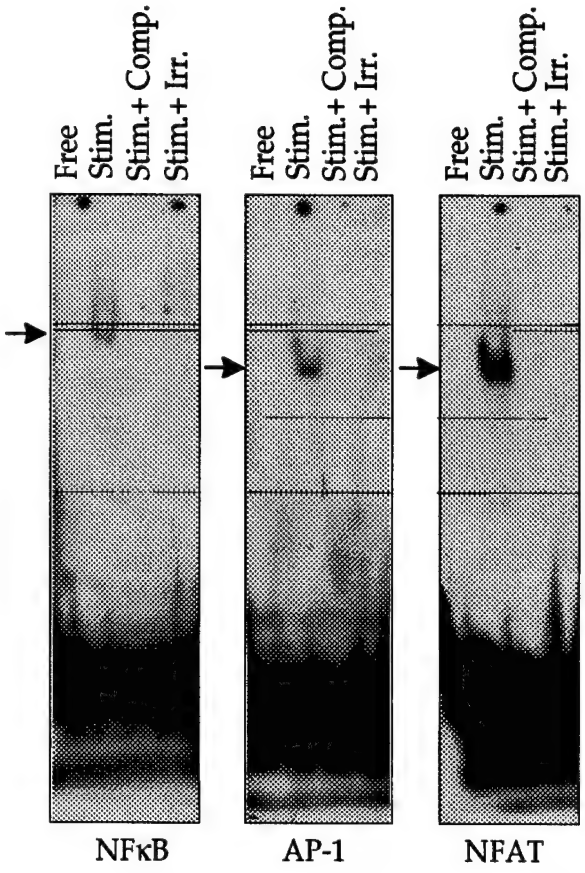

Figure 6. Suppression of DNA-binding activities by irradiation in activated B lymphocytes. B cells were pre-treated with irradiation (Stim.+Irr., for 5 minutes at 6 Gy and then incubated for 2 hours in fresh medium). Cells were then stimulated with pre-coated anti-μ antibodies for 6 hours. In addition, control cultures of untreated cells were stimulated with pre-coated anti-μ antibodies for 6 hours (Stim.). Nuclear extracts were prepared and 10 μg of protein was incubated with the indicated ^{32}P-labeled DNA sequence and electrophoresed. The lanes were loaded with DNA without nuclear extract (Free), DNA with extract from untreated and stimulated cells (Stim.), same as Stim. + 50x excess of unlabeled probe (Stim.+Comp.), and DNA with extracts from pre-treated cells that where also stimulated (Stim.+Irr.). The gels were dried and autoradiographed. The arrows mark the specific DNA-protein complexes.

Transcription factor-directed CAT expression studies in transfected cells

Binding to DNA is a prerequisite for the ability of transcription factors to enhance gene expression. Consequently, suppression of DNA-binding should result in inhibition of the expression of genes regulated by specific transcription factors. Therefore, plasmids containing a reporter gene (CAT) and NFκB or AP-1 sequences, were transfected into epithelial cells (as a model system). CAT can only be expressed if the transfected cells express the NFkB or AP-1 transcription factors, respectively. The transfected cells were then stimulated by interleukin-1β for 24 hours. As expected[31], this treatment resulted in enhanced expression of AP-1- and NFκB-directed CAT expression ($P<0.005$), above the basal levels in the epithelial cells (Figure 7). However, when the cells were pretreated with 200 μM H_2O_2 prior to the stimulation with interleukin-1, the levels of AP-1-directed ($P<0.0005$) and NFκB-directed ($P<0.005$) CAT expression were significantly reduced. While hydrogen peroxide pretreatment brought down the NFκB-directed CAT expression to the basal level in untreated cells, the AP-1-directed CAT expression was reduced to levels that are significantly ($P<0.0005$) below those in untreated cells.

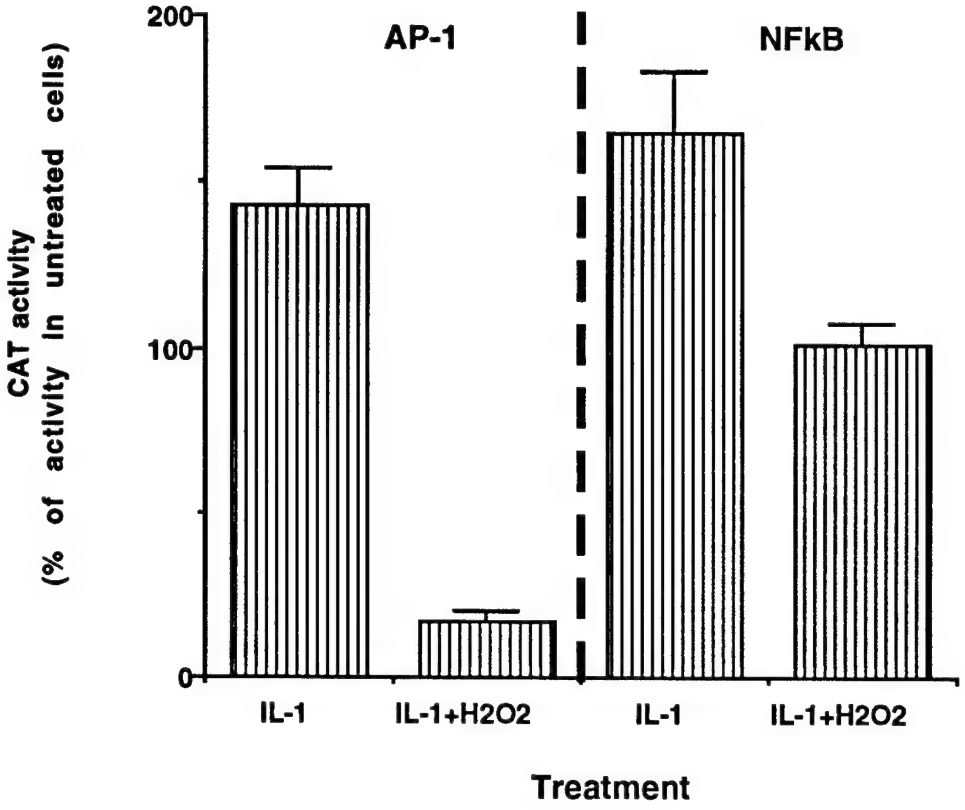

Figure 7. Suppression of transcription factor-directed reporter gene (CAT) expression in stimulated cells pretreated with hydrogen peroxide. A549 cells were transfected with plasmids containing AP-1- or NFkB-directed CAT constructs. After 24 hours, the cells were treated with human recombinant interleukin-1β (R&D Systems, Minneapolis, MN) at 20 ng/ml for 24 hours (IL-1). Other aliquots of transfected cells were treated (24 hours after transfection) for 2 hours with hydrogen peroxide, washed and incubated in fresh medium for 2 hours, and then treated with interleukin-1 at 20 ng/ml for 24 hours (IL-1+H2O2). Cells were then washed and lysed, protein concentration was determined and CAT activity was assayed by ELISA. Every sample was co-transfected with a β-gal plasmid, to control for transfection efficiency, and β-gal activity in the cell lysates was also determined by ELISA. Results are expressed as percentage of CAT activity in transfected but untreated cells, for each CAT plasmid respectively.

Lipid peroxide determination in lymphocytes exposed to oxidative stress

Since the goal of these studies was to develop markers of oxidative stress in lymphocytes, we measured lipid peroxidation as a biochemical parameter of exposure to oxidants. The basal level of lipid peroxides in T lymphocytes was 2.4±0.7 nmoles lipid hydroperoxides/mg protein. Only treatment with hydrogen peroxide at 20 μM for 2 hours induced a rise in cellular peroxides to 11.9±1.8 nmoles lipid hydroperoxide/mg protein ($P<0.0005$), while the other treatments (hydrogen peroxide at 50-200 μM, polyamine oxidase and irradiation) did not induce any rise in the levels of lipid peroxides above basal levels.

Measurements in B lymphocytes did not show enhancement of cellular peroxides by any of the above-mentioned oxidative stresses.

Studies benefiting from the infra-structure supported by this grant

Up to here, we described the results of research that was directly funded by this grant. The infrastructure created in our laboratory (including the purification of blood cells, maintaining of an epithelial cell line and establishment of the electrophoretic mobility shift assay) allowed the graduate students of the principal investigator to perform their separate research activities. Although these activities were in no way directly supported by this grant, it is appropriate to recognize the fact that the students' research (which is ancillary to the grant) benefited indirectly from the grant. Describing these results is further justified by the general areas of the students' projects: the signal transduction cascade initiated in lung cells by the oxidant ozone; and cellular stress in transformed lymphocytes.

Ozone is a major environmental oxidant which is generated by UV radiation and high-voltage sparks. The oxygen radicals it generates are similar to those encountered by tissues during reperfusion. We found that the lung inflammation induced by this oxidant is mediated through the secretion of the neutrophil chemotactic factor - IL-8. The lung epithelial cells respond directly to ozone. Consequently, a signaling cascade is initiated in which oxidative stress and protein tyrosine kinases, as well as cAMP-dependent kinases, transmit the signal from the plasma membrane to the nucleus. There, several transcription factors including NFκB, NF-IL-6 and AP-1, enhance the expression of a set of genes including IL-8 (Appendix A). The ensuing biosynthesis and secretion of this cytokine results in neutrophil recruitment to the alveolar lumen and in other characteristics of lung inflammation. Identification of the cellular events necessary for ozone-induced lung inflammation to occur, may allow the development of means to control and suppress this histopathological response.

Apoptosis is a form of active cell death which occurs in response to severe cellular stresses including oxidative stress. There are instances where clinicians are interested in inducing this "suicide" path in cells. A prominent example is the death of cancer cells. Many anti-cancer drugs kill their target cells by inducing apoptosis. Unfortunately, cancer cells have developed an array of mechanisms to avoid death, including the activity of an efflux pump (P-glycoprotein=P-gp) that removes anti-cancer drugs from intra-cellular compartments. To counteract this defensive response, inhibitors of P-gp have been found that enhance the effectiveness of anti-cancer drugs. We studied the ability of three P-gp inhibitors (verapamil, MRK16-a monoclonal antibody against P-gp, and PSC 833-a non-immunosuppressive derivative of cyclosporin D) to kill human T lymphoma cells directly, by inducing apoptosis. Based on morphological and biochemical criteria, P-gp inhibitors induced apoptosis in transformed T lymphocytes but not in their normal peripheral blood counterparts (Appendix B). PSC 833 At the same concentrations is currently used in advanced clinical trials as a modulator of drug resistance. Since anti-cancer drugs were not present in our experimental cultures we conclude that transformed T cells require P-gp to maintain viability, and that the enhancement of anti-cancer drugs efficacy by P-gp inhibitors may be partially explained by the ability of the latter to directly kill lymphoma cells.

The transcription factor NF-IL-6 enhances the expression of the gene (MDR1) that encodes P-gp. Since the expression of NF-IL-6 is regulated by protein kinase C (PKC), and we formerly found that non-steroidal anti-inflammatory drugs (NSAID) induce PKC activity in human T lymphocytes, we assessed the ability of NSAID to enhance P-gp expression and function. Aspirin and sodium salicylate, at plasma attainable levels, induced the expression of NF-IL-6 DNA-binding, P-gp mRNA, protein and function, in a human T lymphoma cell line

(Appendix C). These results raise the possibility that the use of NSAID may be contra-indicated during cancer chemotherapy sessions.

Discussion

Three modes of oxidative stress: hydrogen peroxide, polyamine oxidase activity generating hydrogen peroxide gradually, and irradiation, suppressed the activation-dependent DNA-binding activities of three transcription factors: NFκB, AP-1 and NFAT, in human peripheral blood T lymphocytes. Only exposure to polyamine oxidase and hydrogen peroxide at 200 μM suppressed the DNA-binding activities of NRE-A and AP-3, respectively. Similar to T cells, the DNA-binding activities of NFκB, AP-1 and NFAT were also suppressed by irradiation in human peripheral blood B lymphocytes. NFkB- and AP-1-directed expressions of the CAT reporter gene were suppressed by hydrogen peroxide exposure. Only exposure to hydrogen peroxide at 20 μM generated measurable lipid peroxidation products in T lymphocytes.

We have previously reported[29] that exposure to polyamine oxidase results in suppression of transmembrane signal transduction in human peripheral blood T lymphocytes. This leads to suppression of the activation-dependent expression of transcription factors in the nucleus, and finally to inhibition of the transcription of the IL-2 gene. In the current study, we compared the effects of different types and levels of oxidative stress on nuclear signal transduction in exposed human lymphocytes. Three transcription factor DNA-binding activities were suppressed in T lymphocytes by every condition of oxidative stress employed, while two transcription factors were mostly unmodulated. Similarly, various in vivo oxidative stresses in rats affected differentially stress-responsive transcription factors and genes[32]. Our results suggest that activation-induced DNA-binding activities of NFκB, AP-1 and NFAT may serve as sensitive markers of oxidative stress in human peripheral blood T lymphocytes. Moreover, these activities respond to either acute or chronic stresses involving hydrogen peroxide as well as hydroxyl radicals. The restricted response of NRE-A DNA-binding to low and chronic stress (polyamine oxidase activity), and the restricted response of AP-3 DNA-binding to high and acute stress (hydrogen peroxide at 200 μM), may allow the use of these activities as markers for the respective specific modes of oxidative stress. The reasons for these restricted responses are unclear and may be related to the signaling pathways leading to the nuclear expression of NRE-A and AP-3.

The DNA-binding activities of NFκB and AP-1 are induced upon exposure to oxidants[33-35]. The apparent contradiction with our results may be resolved by recognizing that in our system (but not in the other studies mentioned), cells were incubated for 2 hours in fresh medium after the exposures, followed by stimulation for 6 hours, and only then were DNA-binding activities determined. Therefore, we are assessing the effects of oxidative stress on T and B cell mitogenic activation rather than the direct effect on transcription factor activities. In this context, the decline in IL-2 production by human T lymphocytes from aged persons is associated with impaired activation of AP-1 and NFAT. In view of the oxygen radical-related theory of aging, this is potentially an example of T cell suppression at the transcription factor level by oxidative stress in vivo[36].

We found that radiation suppressed DNA-binding activities in activated T and B lymphocytes. Radiation of human lymphocytes in vitro was previously found to suppress constitutive surface marker expression[37] and enhance micronuclei occurrence following

stimulation[38]. Our results suggest DNA-binding activities as measures of functional suppression of lymphocyte activation by radiation.

An epithelial cell transfection model system was used to assess the functional implications of DNA-binding suppression by oxidative stress. The expression of the CAT reporter gene linked to either NFκB or AP-1 sequences, was enhanced by stimulation with IL-1 and suppressed by hydrogen peroxide pre-exposure. Thus, oxidative stress suppresses not only the ability of transcription factors to bind their cognate DNA sequences, but also their ability to activate gene expression. Since NFkB and AP-1 were recently found to be the most important IL-2 cis-regulatory elements in normal T cells[39], our results strongly suggest that suppression of transcription factor function contributes to the down-regulation of IL-2 production and of cellular activation[29] caused by inducing oxidative stress in human lymphocytes.

Direct measurements of lipid hydroperoxides did not detect increased levels following oxidative stress, except for exposure of T lymphocytes to the lowest concentration of hydrogen peroxide. A possible explanation to these findings is that the direct biochemical damage was repaired within two hours after the exposures. The lowest concentration of hydrogen peroxide may not have been sufficient to induce appropriate levels of anti-oxidant defenses, allowing the lipid peroxidation to be detected. Experiments to determine the levels of anti-oxidant molecules in oxidatively-stressed lymphocytes have been commenced. On the other hand, all 3 modes of oxidative stress resulted in suppression of cellular function that was clearly evident even 8-26 hours after the exposures, as judged by transcription factor activities. We have previously found that oxidative stress suppresses early signal transduction steps, protein tyrosine phosphorylation and calcium mobilization[29]. Therefore, the eventual suppression of transcription factor activities may actually reflect early effects of oxidative stress on lymphocyte transmembrane signal transduction. These effects are apparently not reversible, at least within 2 hours after the stress.

CONCLUSIONS

We proved our hypothesis that oxidative stress alters the expression of transcription factors in human lymphocytes. Oxidative stress suppressed the activation-dependent DNA-binding activities of three transcription factors: NFκB, AP-1 and NFAT, in human peripheral blood T lymphocytes. Similar to T cells, the DNA-binding activities of NFκB, AP-1 and NFAT were also suppressed by irradiation in human peripheral blood B lymphocytes. NFkB- and AP-1-directed expressions of the CAT reporter gene were suppressed by hydrogen peroxide exposure. These results suggest that transcription factor functions, both binding to cognate DNA sequences and activation of a cis reporter gene, can be used as markers of blood lymphocyte exposure to oxidants. Several modes of oxidative stress were studied, including low level longitudinal stress mimicking exposures to environmental chemical toxicants. Since the transcription factors under study are essential for optimal expression of IL-2, and therefore indirectly also for successful activation and function of the cellular immune response, suppression of these nuclear signaling events reflects not only exposure but also the detrimental effect of exposure to oxidative stress inducers. These studies were conducted with human peripheral blood lymphocytes which are readily available, and should therefore be amenable to development into population-based markers of environmental exposure to oxidants.

Only exposure to hydrogen peroxide at 20 μM generated measurable lipid peroxidation products in T lymphocytes while lipid peroxidation was undetectable in B lymphocytes exposed to any of the modes of oxidative stress under study. These were unexpected results since we assumed that lipid peroxidation, being a biochemical hallmark of cellular oxidative stress, will occur upon exposure of lymphocytes to oxidants. A possible explanation may be that anti-oxidant defenses are mounted by stressed lymphocytes, which repair the structural damage. On the other hand, the damage to signal transduction in activated lymphocytes is sufficient to persist in spite of the cellular anti-oxidative defenses. Therefore, our results suggest that a functional parameter (nuclear signal transduction) is much more sensitive than a structural parameter (lipid peroxidation) as a marker of oxidative damage to human blood lymphocytes.

Military occupational settings include the exposure of soldiers to smoke and combustion products which contain very high concentrations of oxidants including peroxides and peroxyl radicals. Also, oxidative damage may occur secondary to biological or chemical weapon exposure. Serious trauma is another instance where the resultant ischaemia and reperfusion can cause oxidative stress and tissue antioxidant depletion.

The vast majority of studies into human T cell signal transduction and gene regulation where carried out using transformed cell lines. We, however, use ex vivo peripheral blood cells which are a physiological target of oxidants and therefore are the most appropriate model to identify toxic hazards, and the only system that allows follow-up of exposed individuals.

The results and conclusions of this project's first year provide three direct benefits:

1. The scientific basis for a quick, inexpensive, in vitro test method (electrophoretic mobility shift assay to determine transcription factor binding to cognate DNA sequences) to identify immunotoxic oxidative hazards and create a health hazard data base of such compounds.
2. This method will allow the identification of personnel exposed and affected by oxidants, by testing their blood.
3. Through the use of this method it will be possible to monitor the success of anti-oxidant prevention/treatment schemes in soldiers exposed to oxidants, or in wounded soldiers suffering of reperfusion-associated oxidative stress.

References

1. Imboden, J. B.; Weiss, A. The initiation of human T lymphocyte activation. *Prog. in Allerg.* **42:**246-279; 1988.

2. McCrady, C .W.; Ely, C. M.; Westin, E.; Carchman, R. A. Coordination and reversibility of signals for proliferative activation and interleukin-2 mRNA production in resting human T lymphocytes by phorbol ester and calcium ionophore. *J. Biol. Chem.* **263:**18537-18544; 1988.

3. Alexander, D. R.; Cantrell, D. A. Kinases and phosphatases in T-cell activation. *Immunol. Today.* **10:**200-205; 1989.

4. Ledbetter, J. A.; Tonks, N. K.; Fisher, E. H.; Clark, E. A. CD45 regulates signal transduction and lymphocyte activation by specific association with receptive molecules on T or B cells. *Proc. Natl. Acad. Sci. (USA)* **85:**8628-8632; 1988.

5. Fugita, T.; Shibuya, H.; Ohashi, T.; Yamanishi, K.; Taniguchi, T. Regulation of human interleukin-2 gene: Functional DNA sequences in the 5' flanking region for the gene expression in activated T lymphocytes. *Cell.* **46:**401-407; 1986.

6. Crabtree, G. Contingent genetic regulatory events in T lymphocyte activation. *Science.* **243:**355-361; 1989.

7. Granelli-Piperno, A.; Nolan, P. I. Nuclear transcription factors that bind to elements of the IL-2 promoter: Induction requirements in primary human T cells. *J. Immunol.* **147:**2734-2739; 1991.

8. Hoyos, B.; Ballard, D. W.; Bohnlein, E.; Siekevitz, M.; Greene, W. C. kB`-specific DNA binding proteins: role in the regulation of human interleukin-2 gene expression. *Science.* **244:**457-460; 1989.

9. Skerka, C.; Decker, E. L.; Zipfel, P. F. A regulatory element in the human interleukin 2 gene promoter is a binding site for the zinc finger proteins Sp1 and EGR-1. *J. Biol. Chem.* **270:**22500-22506; 1995.

10. Williams, T. M.; Moolten, D.; Burlein, J.; Romano, J.; Bhaerman, R.; Godillot, A.; Mellon, M.; Rauscher III, F. J.; Kant, J. A. l. Identification of a zinc finger protein that inhibits IL-2 gene expression. *Science.* **254:**1791-1794; 1991.

11. Singh, H. Genetic analysis of transcription factors implicated in B lymphocyte development. *Immunol. Res.* **13:**280-290; 1994.

12. Huo, L.; Rothstein, T. L. Receptor-specific induction of individual AP-1 components in B lymphocytes. *J. Immunol.* **154:**3300-3309; 1995.

13. Francis, D. A.; Karras, J. G.; Ke, X-Y.; Sen, R.; Rothstein, T. L. Induction of the transcription factors NF-κB and NF-AT during B cell stimulation through the CD40 receptor. *Int. Immunol.* **7:**151-161; 1995.

14. Yaseen, N. R.; Maizel, A. L.; Wang, F.; Sharma, S. Comparative analysis of NFAT (nuclear factor of activated T cells) complex in human T and B lymphocytes. *J. Biol. Chem.* **268:**14285-14293; 1993.

15. Cochrane, C. G. Cellular injury by oxidants. *Amer. J. Med.* **91:**3C-23S-3C-30S; 1991.

16. Simpson, J. A.; Narita, S.; Gieseg, S.; Gebicki, S.; Gebicki, J. M.; Dean, R. T. Long-lived reactive species on free-radical-damaged proteins. *Biochem. J.* **282:**621-624; 1992.

17. Thompson, A. M. The oxidizing capacity of the earth's atmosphere: Probable past and future changes. *Science.* **256:**1157-1165; 1992.

18. Menzel, D. B. Ozone: an overview of its toxicity in man and animals. *J. Toxicol. Environ. Health.* **13:**183-204; 1984.

19. Peterson, M. L.; Rommo, N.; House, D.; Harder, S. In vitro responsiveness of lymphocytes to phytohemmagglutinin. *Arch. Environ. Health.* **33:**59-63; 1978.

20. Peterson, M. L.; Smialowicz, R.; Harder, S.; Ketcham, B.; House, D. The effect of controlled ozone exposure on human lymphocyte function. *Env. Res.* **24:**299-308; 1981.

21. Orlando, G. S.; House, D.; Daniel, E. G.; Koren, H. S.; Becker, S. Effect of ozone on T-cell proliferation and serum levels of cortisol and beta-endorphin in exercising males. *Inhalation Toxicol.* **1:**53-63; 1988.

22. Pryor, W. A.; Das, B.; Church, D. F. The ozonation of unsaturated fatty acids: Aldehydes and hydrogen peroxide as products and possible mediators of ozone toxicity. *Chem. Res. Toxicol.* **4:**341-348; 1991.

23. Tepper, J. S.; Wiester, M. J.; Weber, M. F.; Fitzgerald, S.; Costa, D. L. Chronic exposure to a simulated urban profile of ozone alters ventilatory responses to carbon dioxide challenge in rats. *Fund. Appl. Toxicol.* **17:**52-60; 1991.

24. Buxton, G. V. Basic radiation chemistry in liquid water. In: Baxendale, J. M.; Buse, F., eds. *The study of fast processes and transient species by electron pulse radiolysis* . Dordrecht: D. Reidel; 1982:241-260.

25. Flescher, E.; Bowlin, T. L.; Talal, N. Polyamine oxidation down-regulates IL-2 production by human peripheral blood mononuclear cells. *J. Immunol.* **142:**907-919; 1989.

26. Burns, F. J.; Sargent, E. The induction and repair of DNA breaks in rat epidermis irradiated with electrons. *Radiat. Res.* **87:** 137-144; 1981.

27. Flescher, E.; Bowlin, T. L.; Ballester, A.; Houk, R.; Talal, N. Increased polyamines may downregulate interleukin 2 production in rheumatoid arthritis. *J. Clin., Invest.* **83:**1356-1362; 1989.

28. Schreiber, E.; Matthia, P.; Muller, M. M.; Shaffner, W. Rapid detection of octamer binding proteins with "mini-extracts", prepared from a small number of cells. *Nucl. Acids Res.* **17:**6419; 1989.

29. Flescher, E.; Ledbetter, J. A.; Schieven, G. L.; Vela-Roch, N.; Fossum, D.; Dang, H.; Ogawa, N.; Talal, N. Longitudinal exposure of human T lymphocytes to weak oxidative stress suppresses transmembrane and nuclear signal transduction. *J. Immunol.* **153:**4880-4889; 1994.

30. Nourooz-Zadeh, J.; Tajaddini,-Sarmadi, J.; Wolff, S. P. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation - Xylenol Orange assay. *Anal. Biochem.* **220:**403-409; 1994.

31. Ray, K. P.; Kennard, N. Interleukin-1 induces a nuclear form of transcription factor NFκB in human lung epithelial cells. *Agents Actions* **38:**C61-C63; 1993.

32. Tacchini, L.; Pogliaghi, G.; Radice, L.; Anzon, E.; Bernelli-Zazzera, A. Differential activation of heat-shock and oxidation-specific stress genes in chemically induced oxidative stress. *Biochem. J.* **309:**453-459; 1995.

33. Schreck, R.; Rieber, P.; Baeuerle, P. A. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J.* **10:**2247-2258; 1991.

34. Schieven, G. L.; Kirihara, J.; Myers, D. E.; Ledbetter, J. A.; Uckun, F. M. Reactive oxygen intermediates stimulate tyrosine phosphorylation in human lymphoid cells, triggering calcium mobilization and induction of NF-κB DNA binding activity. *Blood* **84:**1212-1220; 1993.

35. Devary, Y.; Gottlieb, R.; Lau, L.; Karin, M. Rapid and preferential activation of the c-jun gene during the mammalian UV response. *Mol. Cell. Biol.* **11:**2804-2811; 1991.

36. Whisler, R. L.; Beiqing, L.; Chen, M. Age-related decreases in IL-2 production by human T cells are associated with impaired activation of nuclear transcriptional factors AP-1 and NF-AT. *Cell. Immunol.* **169:**185-195; 1996.

37. Rowley, D. A.; Kelley, W. A.; Manders, J. H. Flow cytometric analysis of lymphocyte surface markers following a 1-Gy dose of gamma radiation. *Aviat. Space Environ. Med.* **64:**528-533; 1993.

38. Holmen, A.; Karlsson, A.; Bratt, I.; Hogstedt, B. Micronuclei and mitotic index in B-, T4- and T8-cells treated with mitomycin C and γ-irradiation. *Mutat. Res.* **309:**93-99; 1994.

39. Hughes, C. C. W.; Pober, J. S. Transcriptional regulation of the interleukin-2 gene in normal human peripheral blood T cells. *J. Biol. Chem.* **271:**5369-5377; 1996.

APPENDIX A

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EFFECTS OF REACTIVE OXYGEN SPECIES ON INTRACELLULAR CALCIUM IN COW TRACHEAL EPITHELIUM

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Reactive oxygen species (ROS) are proposed to play an important role in the oxidative stress-induced epithelial cell dysfunction or injury. We studied the effects of ROS on intracellular cytosolic levels of free calcium ($[Ca^{2+}]_i$) by spectrofluorimetry with fura-2 in cultured epithelium from cow trachea. Successive application of hypoxanthine (5×10^{-4}M) and xanthine oxidase (HX-XO), which generates superoxide anion (O_2^-) and H_2O_2, increased $[Ca^{2+}]_i$ in a dose-dependent manner. This $[Ca^{2+}]_i$ response was biphasic, consisting of an initial transient peak within one minute and a following sustained response. HX-XO (20 mU/ml), which generated 5 nmol O_2^-/ml/min as determined by cytochrome C method, increased $[Ca^{2+}]_i$ from 110 ± 13 to 248 ± 29 nM (mean ± SE, $p<0.05$). This effect was reduced in the presence of superoxide dismutase (SOD) (200 U/ml), catalase (CAT) (200 U/ml), and SOD plus CAT by 29%, 43% and 50% respectively. In addition, allopurinol (3mM), an XO inhibitor, likewise inhibited the HX-XO action by 84%. These findings indicate a role of both O_2^- and H_2O_2 in the observed $[Ca^{2+}]_i$ response, but exogenously applied H_2O_2 or glucose oxidase produced only a small effect on $[Ca^{2+}]_i$. On the other hand, pretreatment of cells with L-N^G-nitroarginine methylester (L-NAME), a specific inhibitor of NO synthase, reduced the HX-XO induced increase in $[Ca^{2+}]_i$ by 61%. These results suggest that : (1) O_2^- and H_2O_2 generated by HX-XO may have different effects on the $[Ca^{2+}]_i$ response, and interaction of ROS contribute to disruption of intracellular Ca^{2+} homeostasis. (2) NO modulate ROS-induced $[Ca^{2+}]_i$ response.

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ALTERNATIVE SPLICING OF INDUCIBLE HUMAN NITRIC OXIDE SYNTHASE mRNA.

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Nitric oxide (NO), an important mediator of physiologic and inflammatory processes in the lung, is a product of nitric oxide synthases (NOS). The inducible NOS isoform (iNOS) is widely expressed in human cells and upregulated by inflammatory mediators (e.g. cytokines). The human iNOS gene, containing 26 exons, encodes a protein of 131 kDa. Alternative mRNA splicing from a single transcript allows for the generation of various forms of mRNA that can be translated into different protein products. These products, which differ from each other by the inclusion or exclusion of specific sequences, may have distinct functions and regulatory properties. This study was aimed at investigating the presence of alternative splicing of human lung iNOS mRNA. Total RNA was extracted from human alveolar macrophages and bronchial epithelial cells, obtained by bronchoalveolar lavage and bronchial brushing, respectively, of normal volunteers. Human lung RNA was purchased from Clontech; Palo Alto, CA. RNA was reverse transcribed and cDNA was analyzed using the polymerase chain reaction (PCR) with specific primers for segmental analysis of the iNOS gene. PCR products were subcloned into plasmid vectors and sequenced. Four sites of alternative splicing were identified which included deletion of: i) exon 5; ii) exons 8 and 9; iii) exons 9,10 and 11; and iv) exons 15 and 16. Deletion of exon 5 (149 bases) leads to a translational frame shift and a premature stop codon in exon 6 predicting a truncated protein. Southern analyses of PCR products were consistent with the presence of the alternatively spliced mRNA transcripts in other human tissues. In A549 (a human alveolar type II epithelium-like lung carcinoma cell line) and DLD1 (a human colorectal adenocarcinoma) cells, iNOS induction by cytokines and lipopolysaccharide was also associated with an increase in mRNA transcripts with exon 5 deletion. Elucidation of the functions of the alternatively spliced human iNOS mRNA transcripts may yield useful information regarding the regulation of nitric oxide synthesis in the lung.

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CYCLIC GUANOSINE MONOPHOSPHATE AS A SECOND MESSENGER IN AIRWAY EPITHELIAL CELLS.

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Increased awareness of the role of nitric oxide in the airway has stimulated research into the role of cyclic guanosine monophosphate (cGMP) as a second messenger in airway epithelial cells. cGMP is a second messenger found in many non-airway epithelial cells, where it may contribute to the control of electrolyte transport. It has been shown to have a second messenger role in cultured nasal epithelial cells (Geary et al, American Journal of Physiology, 1993: 9; L598-605) but its role in lower airways is unknown. We have studied the cGMP system in ovine tracheal epithelial cells. Epithelial cells were removed from tracheas obtained from abattoir specimens and incubated with activators of soluble and membrane bound guanylate cyclase. cGMP was measured using an ELISA method (Amersham™). Sodium nitroprusside, a nitric oxide donor and activator of guanylate cyclase, elevated cGMP levels by up to 25 times baseline values in a dose dependent manner (10^{-7} M to 10^{-3} M). Brain natriuretic peptide and C-type natriuretic peptide (both 10^{-10} M to 10^{-6} M), both activators of membrane bound guanylate cyclase did not significantly elevate cellular levels of cGMP. These results suggest that ovine tracheal epithelial cells contain a soluble but not a membrane bound guanylate cyclase. This systems role as a controller of epithelial cell biology is currently under investigation.

UK Cystic Fibrosis Trust

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INHIBITION OF PROTEIN PHOSPHORYLATION PREVENTS THE OZONE-INDUCED DNA-BINDING ACTIVITY OF NF-KB, NF-IL6, AND AP-1 IN AIRWAY EPITHELIAL CELLS.

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Ozone, the major oxidant in photochemical smog, induces inflammation in the lower airways. The epithelium, one of the first targets inhaled ozone will encounter, could contribute to the ozone-induced airway inflammation through the release of IL-8, a potent neutrophil chemotactic factor. The expression of IL-8 is under the control of the transcription factors NF-KB, NF-IL6, and AP-1. Protein phosphorylation is a mechanism by which DNA-binding activity of these transcription factors can be induced. A human type-II-like epithelial cell line (A549, ATCC, Rockville, MD) was grown to a monolayer in Costar Transwells (Costar, Cambridge, MA) and exposed *in vitro* to O_3/air. Induction of DNA-binding activity was assessed by electrophoretic mobility shift assay. The DNA-binding activity of NF-KB, NF-IL6, and AP-1 was induced in A549 by exposure to 0.1 ppm O_3 for 5 hours. The presence of 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7), an inhibitor of PKC and cAMP-dependent kinases, in the basolateral buffer during exposure, prevented the induction of DNA-binding activity of NF-KB, NF-IL6, and AP-1. IL-8 levels measured 48 hours after exposure were increased in O_3-exposed A549 cells, as compared to air-exposed cells. These results suggest that exposure of airway epithelial cells to ozone activates a signaling cascade in which protein phosphorylation preceeds the activation of NF-KB, NF-IL6, and AP-1. These transcription factors, in turn, enhance the expression of the IL-8 gene. (Sponsored by EPA R819342 and DAMD17-95-1-5058).

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BOVINE TRACHEAL RESPONSIVENESS *IN VITRO*: NO EVIDENCE FOR A ROLE OF NITRIC OXIDE.

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Nitric oxide (NO) induces smooth muscle relaxation and is involved in the responsiveness of airways from humans and some animal species. We have developed a method to standardize bovine tracheal smooth muscle strips for measuring airway responsiveness in vitro (*J. Pharmacol. Toxicol. Meth.*, 34:103-108, 1995). Tracheal smooth muscle was cut into strips 2.5-mm thick and 10-mm long. A preload of 5 g caused the highest contraction and relaxation of the tissues in response to pharmacological agents. Moreover, at a preload of 5 g the variations among the pD_2 values and maximal responses induced by histamine, methacholine, KCl and salbutamol were <7%. The effects of the NO precursor, L-arginine (L-Arg), and the competitive inhibitors of the enzyme NO synthase, N^G-nitro-L-arginine methyl esther (L-NAME) and N^Gmonomethyl L-arginine (L-NMMA), on the histamine responsiveness of isolated bovine tracheal smooth muscle were studied. Incubation of tracheal smooth muscle with L-Arg (1 mM) or L-NAME (120 μM) for 25 min did not influence either the maximal response (E_{max}) or the pD_2 value of histamine. However, L-NMMA (120 μM) caused a hyporesponsiveness to histamine ($P<0.01$). L-Arg did not relax the intact strips precontracted by histamine. In contrast, the direct NO donor, S-nitroso-N-acetyl-penicillamine (SNAP, 1 mM), reversed the contraction induced by histamine completely, indicating the presence of a NO-sensitive system system. In conclusion, NO might be synthesized by the smooth muscle or epithelium of bovine trachea in concentrations too low to exert an effect on histamine-induced contraction. The SNAP-induced relaxation demonstrated that there is a NO/cGMP pathway in bovine trachea.

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EXPRESSION OF TRANSFORMING GROWTH FACTOR-ß1 IN HYPEROXIC MOUSE LUNGS.

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Transforming growth factor -ß1 (TGF-ß1) is a multifunctional cytokine whose activities include regulating cell proliferation, production of extracellular matrix and recruitment of inflammatory cells. Because hyperoxic exposure results in cellular changes that may be mediated by TGF-ß1, the cellular localization of TGF-ß1 was determined in adult C57Bl/6J mice exposed to >95% oxygen. Immunohistochemical analysis of control lungs using an antibody that recognizes mature TGF-ß1 revealed weak immunopositive activity in cuboidal airway cells, platelets and alveolar macrophages. Mice exposed to oxygen for 24 hours had significantly increased staining in cuboidal Clara cells of the airway, but not ciliated cells. Staining was also observed in granulated cells of the parenchyma suggestive of type II cell localization. Northern blot analysis revealed that TGF-ß1 mRNA levels were not markedly altered at this time. The only observable histologic change was a thickening of the airway in oxygen exposed lungs. These findings suggest that short-term oxidant exposure results in a post-transcriptional increase in TGF-ß1 that is cell-type dependent. Funded by Strong Children's Research Center.

This abstract is funded by:

APPENDIX B

From: FASEB J. 10:A1071, 1996.

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INTRACELLULAR PEROXIDE GENERATION AS AN EARLY EVENT BY SOME BUT NOT ALL APOPTOTIC STIMULI IN T LYMPHOCYTES. Mark S. Williams and Pierre A. Henkart. Experimental Immunology Branch, NCI, NIH Bethesda, MD 20892.

The role of reactive oxygen intermediates (ROI) in apoptotic death has been controversial, although ROI production has been directly shown in some apoptotic death systems. Inhibition of Fas dependent killing in Jurkat or U937 cells by thiol antioxidants (e.g. glutathione) prompted further analysis of oxidants in models of T cell death using the oxidation sensitive dye chloromethyldichlorofluorescin diacetate (CM-DCFH). Incubation of Fas bearing Jurkat cells or a T cell hybridoma with anti-Fas induced formation of ROI within 1 hr in a concentration and time dependent manner, with nearly a 10 fold increase in fluorescence by 3 hr. ROI production occured prior to or concomitant with other measures of apoptotic death and was inhibited by thiol antioxidants which also inhibited death under these conditions. In addition, the ICE-like protease inhibitor ZVAD-FMK, but not the control reagent ZFA-FMK, completely inhibited both ROI production and death in Jurkat cells. Ceramide, a putative second messenger for Fas signaling, and staurosporine also induced apoptotic death and the early formation of ROI in a concentration and time dependent manner. In contrast, neither etoposide nor AraC, both inducers of apoptotic death, induced detectable ROI. The data suggest that ROI play a functional role in some T lymphocyte apoptotic death pathways but are not a common downstream death effector in apoptosis.

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THE CLONING AND IDENTIFICATION OF TWO NOVEL ISOFORMS OF HUMAN ICE. J. He, J. Wu and J. D. Mountz. Univ. of Alabama at Birmingham and Birmingham VAMC, Birmingham, AL 35294.

Human interleukin-1β converting enzyme (ICE) is a cytoplasmic cysteine protease that processes the 31 KDa inactive precursor of interleukin-1β into the 17 KDa active pro-inflammatory cytokine IL-1β. To understand the mechanism of ICE-activation in apoptosis, we established *in vitro* long-term T cell culture from freshly isolated PBMCs of lupus patients or normal family members. We analyzed the expression of ICE mRNA by the RT-PCR technique. This resulted in the identification and cloning of two new alternatively spliced ICE mRNA isoforms and were designated as ICE-2a and ICE-2b. The 5′ end of ICE-2a and ICE-2b (from coding sequence bp 1 to 274) are 100% homologous to ICE-α and ICE-β. The 3′ end of ICE-2a and ICE-2b are identical, but only have 85% homologous to human ICE exon 4. In ICE-2a, there were 62 bp sequence (bp 275 to 337) which is deleted in ICE-2b, is not homologous to any known ICE sequence, instead they are 68% homologous to ZYMV polyprotein (p1 protease). Interestingly, the ICE-2a and ICE-2b are highly expressed in the freshly isolated PBMC, but disappeared after stimulation with PHA/IL-2. These observations suggested that ICE-2a and ICE-2b may play an important role in the regulation of programmed cell death in humans.

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INHIBITION OF PHYTOHEMAGLUTININ (PHA)-INDUCED-APOPTOSIS IN A HUMAN T CELL LINE BY HUMAN HOMOLOGUE OF BACULOVIRUS IAP GENES. I. Vitte-Mony, P. Liston, K. Tamai, R. G. Korneluk and F. Diaz-Mitoma (SPON: I. Lemaire). Lab. of Virology, Children's Hosp. of Eastern Ontario and Apoptogen, Univ. of Ottawa, Ottawa, ONT, K1H 8L1, Canada.

We have investigated the function of three newly described human genes in the Jurkat cell line. These genes *xiap, hiap1* and *hiap2* have homology to both the human *naip* gene and the baculovirus genes *cp-iap and op-iap*, which are inhibitors of apoptosis. After overnight stimulation with PHA, 40-50% of Jurkat cells underwent apoptosis, as determined by propidium iodide staining by FACS analysis. Results confirmed by the observation of DNA laddering. Western blot analysis of these cells showed a concomitant down-regulation of XIAP expression. The Jurkat cells were then transfected with pCDNA3 plasmid containing *myc-tag* alone or with *xiap, hiap1, hiap2*, and stable transfected clones were isolated. After overnight stimulation with PHA, apoptosis was inhibited in the *xiap, hiap1 and hiap2* clones but not in the control. The percentage of inhibition was more significant in the *xiap* clone (inhibition 70-80% after 12-18h incubation) than in the *hiap1 and hiap2* clones (inhibition: 40-50% after 12-18h incubation). These data suggest a role, in immune cells, for this class of new human anti-apoptotic genes in the intracellular mechanism of apoptosis.

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SIGNALS THROUGH 4-1BB PREVENT ACTIVATION-INDUCED CELL DEATH OF PREVIOUSLY ACTIVATED SPLENIC T CELLS. José C. Hurtado, Young-J. Kim and Byoung S. Kwon. Indiana Univ. Sch. of Med., Indianapolis, IN 46202

The expression of the T-cell antigen 4-1BB is induced and sustained during the course of primary T-cell activation. Previously, we and others showed that costimulatory signals relayed through 4-1BB enhanced proliferative T-cell responses; furthermore, blocking the interaction of 4-1BB with its ligand resulted in decreased responses to polyclonal activators and to alloantigens. Recently much attention has been devoted to the study of apoptosis and its relevance in the process of lymphocyte biology. In particular, several groups are actively engaged in elucidating the significance and underlying mechanisms of the activation-induced cell death (AICD) that is observed after T-cell receptor (TCR) crosslinking of previously activated T cells. In the present work we analyzed the role of signaling through 4-1BB in the outcome of AICD induced by TCR reengagement. For the purpose, previously activated splenic T cells were stimulated with anti-CD3 in the presence of costimulatory signals provided with an anti-4-1BB monoclonal antibody. In this report we present evidence that in the presence of such costimulatory signals, previously activated T cells respond to TCR crosslinking with strong proliferative responses and cytokine production; moreover, the characteristic DNA fragmentation and ensuing apoptotic cell death observed after TCR reengagement are significantly reduced as well. A comparison with signaling through the prototypic costimulatory molecule CD28 is presented.

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4-1BB ASSOCIATES WITH A CYTOSOLIC PROTEIN CONTAINING LEUCINE-RICH REPEATS. Ihn-K. Jang and Byoung S. Kwon. Indiana Univ. Sch. of Med., Indianapolis, IN 46202.

4-1BB is a member of tumor necrosis factor receptor (TNFR) superfamily. 4-1BB was not only induced upon T cell activation, but also remains on activated T cells. The natural ligand for 4-1BB (4-1BBL) was also induced and was found on activated antigen presenting B cells and macrophages. We have shown that 4-1BB was a costimulatory molecule and blocking 4-1BB/4-1BBL inhibited T cell activation. We hypothesized that there are proteins associated with the cytoplasmic tail of 4-1BB ($4\text{-}1BB_{cy}$) and that those proteins are involved in the 4-1BB-mediated signal transduction. To identify proteins associated with $4\text{-}1BB_{cy}$, the yeast-based two-hybrid system was used. Because 4-1BB is induced upon T cell activation, it is possible that certain proteins associated with 4-1BBcy might be induced as well. Therefore, a human T cell line was stimulated with PMA and ionomycine to induced 4-1BB and then 4-1BB was cross-linked to trigger the signaling pathway. cDNA library was prepared from such activated T cells in GAL4-activation domain vector. Upon screening the cDNA library, two cDNA clones which demonstrated specificity of interaction with $4\text{-}1BB_{cy}$ were isolated; one encoded TRAF-2 and the other encoded a novel protein, hence we named it as 4-1BBbp-LRR. The 4-1BBbp-LRR comprised of 414 amino acids with two potential N-linked glycosylation sites. There were four motif of leucine-rich repeats (LRR) in the middle of the protein. The LRR was followed by a cysteine-rich region. Binding assays indicated that the cysteine-rich region was responsible for binding to $4\text{-}1BB_{cy}$.

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INHIBITORS OF P-GLYCOPROTEIN (P-gp) FUNCTION INDUCE APOPTOSIS IN TRANSFORMED T LYMPHOCYTES. E. Fleischer, K. Salnikow, J. Azare, H. Tripoli and D. Cohen. NYU Med. Ctr., Tuxedo, NY 10987 and Sandoz Res. Inst., East Hanover, NJ 07936.

Inhibitors of the P-gp efflux pump enhance the cytotoxic effect of anti-cancer drugs (ACD) by preventing active removal of the latter from the cell. This results in many cases in enhanced levels of ACD-induced apoptosis. The objective of this study was to assess the ability of P-gp inhibitors to directly induce apoptosis in human T lymphoma cells. Apoptosis was assessed by flow cytometry, by incorporation of radiolabeled ATP into fragmented DNA, and by microscopy. P-gp inhibitors, Verapamil (V, 50-100 μM) and PSC 833 (P, 0.25-2 μM, a non-immunosuppressive cyclosporin D derivative) inhibited P-gp-mediated rhodamine 123 efflux and induced apoptosis (after 5 hours incubation) in Molt-4 T lymphoma cells, in a dose dependent fashion. For example, P at 1 μM induced apoptosis in 57.8% of the cells. The range of concentrations of P used in this study include levels present in the blood of patients treated experimentally with this drug. In contrast to Molt-4 cells, V and P at the concentrations mentioned above did not induce apoptosis in either quiescent or activated (preincubated with PHA+TPA for two days) human peripheral blood (PB) T lymphocytes. The P-gp inhibitors did not induce apoptosis in PB T lymphocytes even when present for 24 hours. In conclusion: a) Molt-4 cells, but not PB T lymphocytes, require a functional P-gp to maintain homeostasis and prevent apoptotic death; and b) P-gp inhibitors may cause selective lymphoma cell death directly, in addition to their ability to enhance the therapeutic potential of ACD. (These studies have been supported by Department of the Army grant #DAMD17-95-1-5058 and by NIOSH grant #OH07125).

APPENDIX C

From: Proceedings of the American Assoc. for Cancer Research, 37:327-328, 1996.

EXPERIMENTAL THERAPEUTICS

#2218 Sunday, April 21, 1996, 1:00–5:00, Poster Section 5

The phosphorylation state of P-glycoprotein does not affect the pumping of calcein-acetoxymethyl ester. Lankelma, J., Wielinga, P.R., Heijn, M., Broxterman, H.J., Westerhoff, H.V.* *Dept Med. Oncology, University Hospital Vrije Universiteit and *Dept Molec. Cellular Biol., Vrije Universiteit, Amsterdam, The Netherlands.*

We have addressed the still controversial issue whether the phosphorylation state of P-glycoprotein (Pgp) controls its pumping rate. A flow-through system was used for sensitive detection of a change of cellular daunorubicin (DNR) accumulation. In all experiments (n = 3) we observed a decrease of DNR accumulation in Pgp expressing KB8-5 cells upon addition of 100 nM phorbol 12-myristate 13-acetate (PMA) to the medium to stimulate phosphorylation of Pgp. However, we could not exclude that the PMA effect was due to an increase of intracellular pH leading to increased passive cellular DNR efflux or to other cellular changes, since similar effects of PMA were found in wild type cells (n = 2). Plasma membrane transport of the Pgp substrate calcein-acetoxymethyl ester (Cal-AM) did not depend on pH. PMA did *not* affect Cal-AM transport by Pgp of KB-V-1 cells (n = 3) or KB8-5 cells (n = 3) nor in drug sensitive KB3-1 cells (n = 3). This study suggests that PMA induced phosphorylation does not change the rate of pumping by Pgp.

#2219 Sunday, April 21, 1996, 1:00–5:00, Poster Section 5

The V-type ATPase inhibitor, N-ethylmaleimide, increases P-glycoprotein drug binding and phosphorylation in multidrug resistant cells. Wang, Y., and Georges, E. *Institute of Parasitology, McGill University, Quebec, CANADA.*

P-glycoprotein (P-gp) mediates the multidrug resistance (mdr) of tumour cell lines selected with lipophilic cytotoxic drugs. It is presently thought that P-gp interacts directly with lipophilic drugs and causes the efflux of structurally dissimilar compounds in an energy-dependent manner. In this study, we have examined the effects of several ATPase inhibitors on P-gp drug binding and phosphorylation in mdr cells. Our results show that short term treatment of mdr cells with NEM resulted in a concentration-dependent increase in P-gp photoaffinity labelling with iodo-azidoparazosin (IAAP) and [^{3}H] vinblastine accumulation. Interestingly, NEM treated plasma membranes did not show a similar increase in IAAP labelling. Comparison of IAAP photolabelled P-gp from intact cells with or without NEM treatment did not show differences in the photolabelled peptides. Thus, differences in the photolabelling of P-gp with IAAP are unlikely due to differences in photolabelling sites. Furthermore, [^{14}C] NEM labelled peptides of P-gp did not co-migrate with IAAP photolabelled peptides. Therefore, the effect of NEM on P-gp photoaffinity labelling is not caused by the direct modification of P-gp drug binding sites. Interestingly, NEM treated mdr cells showed a dramatic increase in P-gp *in vivo* phosphorylation. Work is in progress to determine if P-gp ATPase, phosphorylation, and drug binding are linked.

#2220 Sunday, April 21, 1996, 1:00–5:00, Poster Section 4

Importance of P-glycoprotein as determinant of chemotherapy response in osteosarcoma. Chan, H.S.L., Grogan, T.M., DeBoer, G., Haddad, G., Ling, V. *Hosp. for Sick Children, Toronto-Sunnybrook Regional Cancer Ctr., Toronto ON M5G 1X8, British Columbia Cancer Ctr., Vancouver BC Canada, Arizona Health Science Ctr., Tucson, AZ.*

Although chemotherapy is pivotal for cure of osteosarcoma (OS), the cause for failure of therapy has not been defined for OS. We investigated the multidrug resistance P-glycoprotein (P170) as the cause for failure of chemotherapy in a retrospective study of 61 OS patients, by assessing if tumor expression of P170 at diagnosis correlated with outcome. P170 was increased in 27 patients but not detectable in 34 at diagnosis, and expression of P170 correlated strongly with outcome. At median followup of 8.9 years, relapse-free (87% vs 0%) and survival rates (94% vs 35%) were significantly higher in patients with P170-negative than positive tumors, irrespective of other prognostic factors ($P < 0.00001$ for both rates after stratifying simultaneously for tumor size, grade and metastasis) and intensity of chemotherapy. For 46 patients treated with preoperative chemotherapy, ≥90% tumor necrosis occurred more frequently with undetectable than increased P170 (48% vs 17%, $P = 0.057$), but an even greater difference was observed in long-term outcomes ($P < 0.00001$ and $P = 0.00002$, comparing relapse-free and survival rates). P170 expression at diagnosis is important as determinant of long-term response to chemotherapy in OS. Patients with P170-expressing tumors are likely to fail and should be considered as candidates for alternative therapy.

#2221 Tuesday, April 23, 1996, 1:00–5:00, Room 30

A systematic selection of variants expressing *mdr*1 mutations affecting steroid transport. Gruol, D.J., Vo, Q.D., Bourgeois, S. *The Salk Institute, La Jolla, CA 92037*

P-glycoproteins transport a wide variety of unrelated compounds. Previous work has demonstrated that mutations affecting the transport of one drug can also alter the transport of other drugs. This phenomenon suggests that there may be a region within P-glycoproteins that can accommodate this diversity by containing many overlapping, but non-identical, drug binding sites. We have developed a selection procedure, involving the murine T-lymphoma line MS23, that allows the isolation of *mdr*1-expressing variants that have completely lost the ability to transport dexamethasone while largely retaining the capacity to transport other drugs. None of the variants characterized so far have altered taxol resistance, but all exhibit diminished puromycin resistance. Thus, the puromycin and steroid binding sites may share common structural elements of the protein. Further characterization has indicated that the putative mutations alter recognition of the steroid's 20-keto group. We are currently identifying the *mdr*1 mutations that are responsible for this phenotype.

#2222 Tuesday, April 23, 1996, 1:00–5:00, Room 30

Intracellular drug sequestration by P-glycoprotein. Shapiro, A.B., Lee, P., and Ling, V. *The British Columbia Cancer Research Centre, Vancouver, B.C., V5Z 1L3, Canada*

In the most widely accepted model for P-glycoprotein (P-gp)-mediated multidrug resistance, P-gp functions as a drug efflux pump at the plasma membrane. Flow cytometry experiments show that highly P-gp-expressing CH^{R}C5 cells accumulate one-sixth to one-ninth the amount of Hoechst 33342 (as measured by fluorescence) as Aux B1 cells, which express very little P-gp. Hoechst 33342 fluoresces only when bound to DNA. During efflux experiments, Hoechst 33342 fluorescence decreased with time in CH^{R}C5 cells faster than in Aux B1 cells. If, however, the chemosensitizer verapamil at 30 μM or cyclosporin A at 5 μM was included to inhibit P-gp during efflux, the Hoechst 33342 fluorescence *increased* with time 3–4.5-fold in CH^{R}C5 cells but not Aux B1 cells, indicating that Hoechst 33342 moved from an aqueous cytoplasmic compartment to the nucleus. Thus about half of the reduction in Hoechst 33342 fluorescence accumulation by CH^{R}C5 cells relative to Aux B1 cells is due to intracellular, P-gp-dependent sequestration of Hoechst 33342 away from the DNA, and the remainder is due to efflux. Confocal immunofluorescence microscopy and immunoelectron microscopy showed that P-gp was present in cytoplasmic vesicles and at the ruffling membrane. These results indicate that P-gp can reduce drug cytotoxicity by sequestering the drug in intracellular, probably pinocytotic, vesicles, as well as by exporting drugs from the cell. Supported by NCI, Canada.

#2223 Monday, April 22, 1996, 10:55–11:10, Room 32

Characterization of *spgp*, a novel P-glycoprotein gene from liver. Childs, S., Yeh R.L., and Ling, V. *British Columbia Cancer Research Centre, 601 W. 10th Ave, Vancouver B.C. V5Z 1L3*

The P-glycoproteins (Pgp's) are a small family of ATP-dependent transporters expressed on the plasma membrane of many cell types. The substrates and functions of these transporters are not completely understood, but the Class I and II Pgp isoforms have been demonstrated to mediate a multidrug resistance phenotype *in vitro* and it is suggested that they play a role in drug resistance in tumours *in vivo*. The Class III Pgp gene on the other hand, is important for normal liver function since it translocates phosphatidylcholine into bile. We have isolated a Pgp gene more distantly related to the known Pgp isoforms, the sister-of-P-glycoprotein or *spgp* gene. The size of the full length gene is estimated to be 5.2 kb, and it has a similar predicted domain arrangement to the other Pgp's. The expression of the *spgp* mRNA is high in the liver. Polyclonal antiserum to a fusion protein has been used to localize the Spgp protein product to the bile canalicular membrane, where Class III Pgp is also expressed. Like the other Pgp isoforms, it is a 170 kD plasma membrane protein. Expression of the *spgp* mRNA is developmentally regulated. In a pattern similar to many liver-specific genes its expression is also down-regulated in hepatocyte culture. The function of Spgp in liver is not known, but its restricted localization suggests an important role in liver transport. Additionally, its similarity to Pgp isoforms suggests it might possibly play a role in drug resistance.

#2224 Tuesday, April 23, 1996, 1:00–5:00, Room 30

P-Glycoprotein stability is regulated by a cell-growth or cell-cycle related mechanism. Zhang, W. and Ling, V. *British Columbia Cancer Research Centre, 601 West 10th Ave., Vancouver, B. C., Canada V5Z 1L3*

Expression of P-glycoprotein (Pgp) is regulated at DNA, mRNA, and protein levels. Regulation of Pgp synthesis and stability at the protein level may be one factor which affects its expression. We showed previously that Pgp is remarkably stabilized when multidrug resistant CH^{R}C5 and SKVCR 2.0 cells are subjected to serum-limited and high density growth conditions while Pgp stability in a leukemia cell line, CEMVLB$_{100}$, is not affected by serum starvation (C. Muller, *et al.* J. Cell. Physiol. 163: 538, 1995). On further analysis we observe that most of CH^{R}C5 and SKVCR 2.0 cells are temporarily arrested at G1 phase while the cell cycle of CEMVLB$_{100}$ is not affected under these growth conditions. The cell cycle and Pgp stability in CH^{R}C5 and SKVCR 2.0 cells revert to normal when serum is added back or density is reduced. Pgp in CEMVLB$_{100}$ is stabilized only when the cell growth is blocked by using amino acid deficient medium. We have also characterized the effects of hormones, growth factors, and irradiation on Pgp expression and stability. Our results suggest that Pgp stability is regulated by a cell-growth or cell cycle-related mechanism. Supported by a grant from NCI of Canada.

#2225 Wednesday, April 24, 1996, 1:00–5:00, Poster Section 12

Non-steroidal anti-inflammatory drugs enhance the expression and function of P-glycoprotein in a human T lymphocyte cell line: Molt-4. Flescher, E., Azare, J., Jaspers, I., Cohen, D.* *New York University Med. Ctr., Tuxedo, NY 10987 and *Oncology Preclinical Res., Sandoz Res. Inst., East Hanover, NJ 07936*

NF-IL-6 regulates MDR1 expression. Since NF-IL-6 expression is regulated by protein kinase C (PKC) activity and previous studies indicated that non-steroidal

anti-inflammatory drugs (NSAID) induce PKC activity in human T lymphocytes, the purpose of the present study was to assess the ability of NSAID to modulate P-glycoprotein expression and function. Molt-4 cells were incubated for 24 hours with aspirin or sodium salicylate at 0.5–2 mM. Using the electrophoretic mobility shift, it was found that aspirin enhanced the DNA-binding activity of NF-IL-6. Furthermore, flow cytometric analysis of the treated cells revealed a dose-dependent increase in the expression of P-glycoprotein, and more than 75% of the cells expressed high levels of P-glycoprotein. Finally, P-glycoprotein function (as measured by Rh123 efflux) was enhanced in a dose-dependent fashion by treatment with NSAID, e.g., sodium salicylate at 1 mM enhanced baseline efflux by 41.1%. This enhancement was fully abolished by the protein kinase inhibitor H-7 at 20 μM. These results suggest that NSAID, probably through induction of intracellular signal transduction, enhance the expression of P-glycoprotein, and ultimately promote more efficient efflux of P-glycoprotein substrates. (These studies have been supported by Department of the Army Grant #DAMD17-95-1-5058 and by NIOSH Grant #OH07125.)

#2226 Tuesday, April 23, 1996, 1:00–5:00, Room 30

Localization of a verapamil binding domain of P-glycoprotein by photoaffinity labeling and site-directed antibodies. Safa, A.R., Agresti, M. *Dept. of Medicine and Cancer Research Center, The University of Chicago, Chicago, IL 60637.*

We synthesized a photoaffinity analog of verapamil (VER), N-(p-azido-[3,5-^{125}I]-salicyl)aminomethyl verapamil ([^{125}I]NAS-VP), and used it to identify the location of its binding site(s) on P-glycoprotein (P-gp). To identify peptides containing VER binding sites, [^{125}I]NAS-VP photolabeled P-gp in MCF-7/Adr human breast cancer cells and SH-SY5Y/VCR human neuroblastoma cells was immunoprecipitated and digested with *S. aureus* V8 protease. Maximum digestion revealed two major 6 and 11 kDa and one minor 12 kDa [^{125}I]NAS-VP-bound peptides. To identify the location of these peptides within P-gp, we produced site-directed antipeptide antisera to several domains of P-gp including peptide sequences located N-terminal or C-terminal to transmembrane domains (TMDs) 6, 11 and 12, respectively. The 6 kDa [^{125}I]NAS-VP-bound domain of P-gp was immunoprecipitated by antisera raised against amino acid residues C-terminal to TMD6, indicating that this VER binding domain is located within or immediately C-terminal to TMD6. The location(s) of the 11 and 12 kDa [^{125}I]NAS-VP-bound domains remain to be found. Interestingly, kinetic data showed a single high affinity [^{125}I]NAS-VP binding site, suggesting that spatially these three peptides may form a single binding site for VER. (Supported by ACS grant DHP-100.)

#2227 Tuesday, April 23, 1996, 1:00–5:00, Room 30

Biogenesis and functional expression of two human P-glycoprotein (MDR1) topological isoforms. Alvina Bragin and William R. Skach, *Department of Molecular and Cellular Engineering, University of Pennsylvania.*

Recent studies have revealed different mechanisms of biogenesis for N- and C-terminus halves of P-glycoprotein. These studies indicate that a predicted cytosolic loop between putative TM segments 8 and 9 resides in the ER lumen in 40–50% of chains. To determine the mechanism directing this topology we engineered MDR1 sequences encoding TM7, and/or TM8 into chimeric cassettes and defined topogenic activities of these sequences in Xenopus oocytes. Our results indicate that TM7 contains both a signal sequence and a separate stop transfer sequence which allow TM7 to span the ER membrane either once or twice. More remarkably, TM8 may span the membrane in either orientation (Type I or Type II topology) by functioning to terminate translocation (e.g. stop transfer sequence) or reinitiate translocation (e.g. signal anchor activity), respectively. Thus TM8 orientation depends on the specific activity of preceding topogenic sequences in TM7. Finally, we show using epitope tagged full length MDR1 that the predicted cytosolic (TM8-9) peptide loop resides in the extracellular space on functional molecules when expressed in mammalian cells. These results demonstrate new complexities in polytopic protein biogenesis and raise important questions regarding the functional role(s) of different MDR1 isoforms and potential mechanisms for directed or regulated expression in different systems and/or cell types.

#2228 Tuesday, April 23, 1996, 1:00–5:00, Room 30

Aureobasidin A, an antifungal cyclic depsipeptide antibiotic, is a substrate for both human MDR1 and MDR2/P-glycoproteins. Kazumitsu Ueda, Yoshitomo Taguchi, Kouji Yamada, Kouichi Kino, and Tohru Komano, *Lab of Biochem, Dept of Agric Chem, Kyoto Univ, Japan.*

The substrates and the physiological functions of the two types of Pgp, MDR1 and MDR2, are thought to be different, although the MDR2 shares about 80% identity at the amino acid level with the MDR1. During our search for peptide antibiotics which interact with human Pgp, we found that *Saccharomyces cerevisiae* expressing human MDR1 showed resistance to aureobasidin A, a new antifungal cyclic depsipeptide antibiotic produced by *Aureobasidium pullulans* R106. When IC_{50} were determined in liquid cultures, yeast cells expressing human MDR1 showed about 4-fold resistance to aureobasidin A. Surprisingly the expression of MDR2 also conferred 2.5-fold resistance to aureobasidin A. The resistance to aureobasidin A conferred by the MDR2 as well as by the MDR1 was overcome by vinblastine, verapamil, and cyclosporin A, depending on the concentrations, but not by colchicine. Aureobasidin A probably interacts directly with Pgps, because it overcame multidrug resistance of human cells expressing MDR1 and inhibited azidopine photoaffinity labeling of MDR1 in human cell membranes. These results suggest that the human MDR1 and MDR2 have conserved domain(s) for drug recognition and share a common substrate for transport.

#2229 Wednesday, April 24, 1996, 1:00–5:00, Poster Section 12

Activation of ERK mitogen-activated protein kinase accompanies phorbol ester-induced but not adriamycin-induced *MDR*1 mRNA expression. Osborn, M.T. and Chambers, T.C. *Dept. Biochemistry and Molecular Biology, University of Arkansas for Med. Sciences, Little Rock, AR 72205.*

Consistent with earlier reports (PM Chaudhary and IB Roninson, Oncol. Res. 4:281; JNCI 85:632), we have found that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (16–32 nM, 16 hr) or adriamycin (350–860 nM, 16–36 hr) induce *MDR*1 expression in two human cancer cell lines, KB-3 and K562. As an initial approach to determine the signaling events involved, we examined the activation of ERK1/2 MAPK in treated cells. ERK activation was assessed by probing immunoblots of cell extracts with antibodies that recognize either the inactive (dephosphorylated) or activated (phosphorylated) forms of ERK. TPA (100 nM, 30 min) caused marked activation of ERK in both KB-3 and K562 cell lines. Adriamycin treatment (up to 860 nM, 30 min), however, failed to activate ERK in either cell line. Thus ERK activation may be involved in TPA-induced but not adriamycin-induced *MDR*1 expression. The effects of PKC inhibition on *MDR*1 induction and ERK activation are now under investigation. The possibility that adriamycin induces *MDR*1 expression via activation of c-Jun NH_2-terminal, or stress-activated, protein kinase is also being examined and the results will be reported. (Supported by American Cancer Society Grant DHP-109.)

#2230 Tuesday, April 23, 1996, 1:00–5:00, Room 30

A mutation in the ninth transmembrane domain (Tm9) of P-glycoprotein (Pgp1) confers a unique multidrug resistance pattern in mammalian cells. Troyer, J.K., Chung, D.T., Melera, P.W. *Program in Molecular & Cell Biology, Dept of Biochemistry & Cancer Center, University of Maryland, Baltimore, MD 21201.*

It has been clearly shown in numerous cell lines that the level of P-glycoprotein (Pgp) expression rises following exposure to chemotherapeutic drugs indicating that Pgp plays an important role in multi-drug resistance. We have previously reported that a double mutation in the sixth transmembrane domain (Tm6) was sufficient to alter Pgp1 cross resistance patterns indicating that this domain plays a vital role in Pgp1 function. In the present study we report that an additional double mutation in the ninth transmembrane domain (Tm9) results in a cross resistance pattern virtually the same as that mediated by the Tm6 mutation. Since mutations in Tm6 and Tm9 are able to confer the same drug resistance patterns, we conclude that both play important roles in the selection and transport of drugs accross the plasma membrane and that both must be considered in any mechanistic model for Pgp1 function.

#2231 Sunday, April 21, 1996, 1:00–5:00, Poster Section 5

Effects of 17β-estradiol and mifepristone (RU486) on p-glycoprotein in the Ishikawa human endometrial cancer cell line. Arango, H., Satyaswaroop, P*., Cavanagh, D., Goldfain, V., Becker, J., *Univ. of South FL, Tampa, Fl 33606 and *Hershey Medical Ctr., Hershey, Pa.*

The effects of 17β-estradiol and the antiprogestin RU486 on the expression of p-glycoprotein in an established human endometrial cancer cell line were studied. Ishikawa cells were cultured in complete medium in the absence or presence of 17β-estradiol (10^{-9}M and 10^{-9}M) and RU486 (10^{-8}M), alone or in combination. Messenger RNA (mRNA) and p-glycoprotein expression were assayed by reverse transcription-polymerase chain reaction, and immunocytochemistry, respectively. Untreated Ishikawa cells express protein and mRNA for p-glycoprotein. Both concentrations of 17β-estradiol inhibited mRNA and expression of p-glycoprotein. RU486 did not have a direct effect on mRNA or protein expression. However, RU486 reversed the inhibition induced by estrogen. These data demonstrate that 17β-estradiol modulates expression of the MDR1 gene in the Ishikawa cell line in a dose dependent fashion. This effect can be overcome by the presence of the antiprogestin RU486. These results demonstrate a previously unrecognized effect of 17β-estradiol on the MRI gene.

#2232 Tuesday, April 23, 1996, 1:00–5:00, Room 30

Drug binding by an N-terminal fragment of human P-glycoprotein (Pgp) distal to transmembrane segment (TM) 4. Castro, A.F. and Altenberg, G.A. *Dept. of Physiology and Biophysics, UTMB, Galveston, Texas 77555-0641.*

The aim of this study was to determine whether or not a fragment of Pgp including TMs 5 and 6, the N-terminal nucleotide binding domain and the minilinker domain binds Pgp substrates. The Pgp fragment was expressed in *E. coli* as a glutathione S-transferase (GST) fusion protein. The fusion protein was photoaffinity labeled with 0.4 μM [^{3}H]azidopine. The binding was specific since verapamil and vinblastine (1–100 μM) reduced the [^{3}H]azidopine labeling of the Pgp fragment. GST was not involved in photolabeling with [^{3}H]azidopine because the Pgp fragment was still labeled when GST was removed by thrombin digestion. These results show that a fragment of human Pgp distal to TM4 includes a drug-binding domain. Because of their hydrophobicity, TMs 5 and/or 6 are most likely to contain the drug-binding site(s).

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